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Effects of monolaurin and lactic acid bacteria starter culture on growth of vegetative cells of *Bacillus cereus* in Iranian white fresh cheese

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Abstract

The harmful effects of many chemical food preservatives are well established, so this has triggered interest in natural methods of preservation. Monolaurin, a monoester of lauric acid, founds naturally in some foods and has various antiviral and antibacterial activities. Evaluation of the effects of monolaurin separately and in combination with lactic acid bacteria (LAB) starter culture on growth of vegetative cells of *Bacillus cereus* ATCC11778 in manufactured cheeses was the purpose of this research. In this study, the number of *B. cereus* in four groups of cheese (C_1: without starter culture and monolaurin, T_1: without starter culture; with monolaurin, C_2: with starter culture; without monolaurin, T_2: with starter culture and monolaurin) was counted on days 0, 1, 3, 5, and 7 of manufacture. In T_1 group, monolaurinin concentrations of 800, 1200, 1600 and 2000 ppm decreased the number of *B. cereus* by 1.2, 2.1, 3 and 3.4 logs, respectively in comparison with C_1 group. InT_2 group with the same concentrations of monolaurin, the number of *B. cereus* in comparison with C_2 group was not significantly affected (p>0.05). In C_2 group, starter culture decreased the number of *B. cereus* by 2.9 logs in comparison with C_1 group. In contrary, the combination of starter culture with monolaurin in T_2 group increased the number of *B. cereus* by 0.6 logs in comparison with C_2 group. Furthermore, in C_2 and T_2 groups by increasing the storage time, the number of *B. cereus* decreased. According to these results, it can be concluded that in cheese samples of T_1 group, monolaurin separately showed the inhibitory effects on the growth of *B. cereus* cells while in cheese samples of T_2 group, the combination of monolaurin with starter culture did not demonstrate the synergistic inhibitory effects on the growth of this bacterium. Therefore, simultaneous use of monolaurin with starter culture is not recommended for improving the microbial shelf-life of Iranian white fresh cheese.

Keywords: Monolaurin, starter culture, *Bacillus cereus*, Iranian white fresh cheese

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Introduction

Iranian white fresh cheese is a soft and unripened cheese in which curd is made mainly through the action of chymosin or other milk-clotting enzymes on milk at pH > 6.2 (Anon, 2002; Madadlou *et al*., 2006 and Rahimi *et al*., 2007).

Bacillus cereus is a gram-positive, spore-forming, motile, rod, and facultative anaerobic bacterium that are widely distributed in nature (Claus and Berkeley, 1986). *B.cereus* can cause food poisoning with diarrheal and emetic syndromes (Kramer and Gilbert, 1989, Granum, 2001). It is a common contaminant in a wide variety of foods, including milk and dairy products, cereals (especially rice), and food additives (Kramer and Gilbert, 1989 and Becker *et al*., 1994). In a survey by Wong *et al* (1988b) on dairy products, 52% of ice creams, 35% of soft ice creams, 29% of milk powders, 17% of fermented milks, and 2% of pasteurized milks and fruit flavored milks were found to be contaminated with *B.cereus*. This bacterium can cause sweet curdling and bitty creamin low-pasteurized dairy products, especially milk and cream (Griffiths, 1992).

Monolaurin (Lauricidin®) is a monoglyceride of lauricacid that has been shown to possess antiviral and antibacterial activity (Kabara, 1993).In addition, a number of fungi, yeasts, and protozoa have been reported to be inactivated by monolaurin (Lieberman *et al.*, 2006). Furthermore, monolaurin has been known as GRAS (generally recognized as safe) and nontoxic food additives (Lieberman *et al*., 2006). In a study by Razavi-Rohani and Griffiths (1994), monolaurin was revealed to be effective against the tested gram-positive bacteria but not against gram-negative bacteria unless in the presence of EDTA (ethylenediamine tetraacetic acid). In a study conducted by Preuss *et al* (2005), monolaurin was shown to be bactericidal against *S. aureus* and *Mycobacterium terrae*, but no tagainst *Escherichia coli* and *Klebsiella pneumoniae*, and also was shown to be bacteriostatic against a variant of the virulent anthrax pathogen, *Bacillus anthracis* Sterne. In another study by Branen and Davidson (2004), EDTA was shown synergistically to enhance the activity of nisin, monolaurin, and lysozyme in tryptic soy broth (TSB) against two enterohemorrhagic *E. coli* strains.

The objective of this study was to determine the effects of monolaurin and lactic acid bacteria starter culture separately and in combination on growth of vegetative cells of B.*cereus* ATCC 11778 in manufactured Iranian white fresh cheese during 7days of storageat +7°C.

Materials and methods

Cow's milk

Pasteurized cow milk was obtained from Iranian Dairy Industries Co., and stored at+4°C. The quality of the milk was within the limits specified in the current Iranian standard for cheese production (2.5% Fat, 8.9% SNF, and pH = 6.7) (Anon, 2002). Milk pasteurization control was done using phosphatase test with Lactognost method (Heyl, Chem.Pharm-Fabrik, and 14167 Berlin). Antibiotic residue was determined using Beta Star kit (Neogen Corporation, Lansing, MI, 48912USA).

Monolaurin

Monolaurin(Med-Chem. Labs, Inc. Galena, IL, USA) tested concentrations (100, 200, 400, 800, 1200, 1600, and 2000 ppm) were prepared through dissolving in96% ethanol (w/v). After filter sterilization (using the syringe with pore size of 0.45 µm), 1 ml of prepared concentrations of monolaurin was added to 1000 ml of pasturized milk.

Bacterial strain and inoculums spreparation

Lyophilized culture of *B.cereus* ATCC 11778 obtained from Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, was used in this study. The lyophilized culture was grown in tube containing 10 ml of BHI broth (Merck KGaA, Darmstadt, Germany), twice, and incubated each time at 30°C for 18 h.

Then it was followed by streaking on BHI agar (Merck KGaA, Darmstadt, Germany) slant and incubated at 30°C for 18 h. The culture was stored at 4°C as working culture and subcultured at monthly intervals. *B.cereus* inoculums were prepared by transferring cells from the working culture to BHI broth. After 18 h incubation at 30°C, second subculture was prepared and incubated for 18 h at 30°C. In a 13 × 100 mm sterile cuvette, the *B.cereus* broth culture was adjusted to optical density of 0.08 at 600 nm, using a PD-303S spectrophotometer (APEL Company, Japan). This adjustment gave a vegetative *B.cereus* cell concentration of 2×10^8 cfuml^{-1}. The numbe of cells in the suspension was estimated by duplicate plating from 10-fold serial dilutions on BHIagar (Merck KGaA, Darmstadt, Germany)and counting the colonies after 24 h incubation at 30°C.

Starter culture

Lyophilized and direct vat set cheese starter culture typeR-704 containing mesophilich fermentative bacteria, *Lactococcus lactis* subsp cremoris and *Lactococcus lactis* subsp lactis (Chr. Hansen's laboratory, FD-DVS CH-1, Denmark) was used to make Iranian white fresh cheese.

Cheese-making procedure

In this study, Iranian white fresh cheese was produced with four different compositions, and each of these was assigned to a group (C_1: without starter culture and monolaurin, T_1: without starter culture; with monolaurin, C_2: with starter culture; without monolaurin, T_2: with starter culture and monolaurin). At first, 1000 ml of pasteurized milk poured into a sterile stainless steel container and the temperature was set to 35°C.Toaccelerate the clotting time or reducing the amount of rennet used [0.01% (w/v)], $CaCl_2$ was added to the milk. Then, 1 ml of *B. cereus* suspension containing approximately10^8 cfuml^{-1} cells was inoculated, so that the final number of bacteria was reached approximately 10^5 cfuml^{-1} of milk. Afterwards, 0.1 ul^{-1}of starter culture was added for C_2 and T_2 cheese groups. Later, 1 ml of prepared concentrations of monolaurin was added and then the milk was mixed using a magnetic stirrer. The milk was kept at 35°C until the pH reached 6.4. Then, rennet CHY-MAX type containing 100% chymosin and 2080 imcug^{-1}(produced by Chr. Hansen's laboratory), was added to achieve the final concentration of 0.001% (w/v). The milk was maintained at 35°C for 1 h to curdle. The curd was cut into cubes of 2 cm^3. After cutting, the curd was allowed to settle for 3 to 5 min and then gently agitated at a gradually increasing rate for 10 min to avoid fusion of freshly cut curd cubes and to facilitate whey expulsion. Following drainage, the curd was placed in stainless steel press for 1 h, to fuse the curd grains into a continuous mass. The molded cheese was cut into 7×7×4 cm and afterwards sterilized. Parchment paper was placed on the surface of the cheese and 3% (w/w) NaCl granules (Merck KGaA, Darmstadt, Germany) were added equally upon the cheese. To absorb the salt and ripening, the cheese was held to 24 h at 23-25°Cand then kept at refrigerator temperature(+7°C) during the 7days of storage (Anon, 2002, Madadlou *et al*., 2006 and Rahimi *et al.*, 2007).

Enumeration and detection of B.cereus

Mannitol phenol red egg yolk poly mix in agar (Merck KGaA, Darmstadt, Germany) was used for the enumeration of *B.cereus* on days 0, 1, 3, 5, and 7 of manufacture. At each sampling period, 10 g of cheese was added to a bottle containing 90 ml of 0.1% (w/v) peptone water and homogenized using a stomacher lab blender for 2 min. Serial 10-fold dilutions of homogenized sample was prepared in sterile peptone water and then surface plated in duplicate on MYP agar, then incubated at 30°C for 24 h. The plates were examined for typical *B. cereus* colonies (rough in texture, dry, pink to purple in color, flattened, irregular and surrounded by a zone of egg yolk precipitate). The number of typical *B. cereus* colonies was expressed as cfu per gram of the sample (Harmon *et al*., 1992).

Statistical analysis

Tests were performed in triplicate. Statistical analyses were done using SPSS version 15.00 for Windows. *B .cereus* counts were converted to $\log_{10}$cfu per gram. To investigate changes in the number of *B .cereus* affected by various concentrations of monolaurin and also the changes in the number of *B. cereus* influenced by different storage days for both groups of cheese (T_1 and T_2), analysis of variance was used (p<0.01, p<0.05). Also differences between the groups were identified with Tukey HSD test. To evaluate the correlation between the number of *B.cereus* affected by monolaurin and storage days in the manufactured cheese samples, Pearson correlation test was used.

Results

The effects of various concentrations of monolaurinfor a definite storage day and also the effects of different storage days for a definite concentration of monolaurin on *B.cereus* count in the four groups of manufactured cheese are given in Table 1. The effect of various concentrations of monolaurin on *B .cereus* count in them anufactured cheese samples (with and without starter culture) is shown in Table 2. In T_1 group, monolaurin in concentrations of 800, 1200, 1600 and 2000 ppm decreased the number of *B. cereus* by 1.2, 2.1, 3 and 3.4 logs, respectively in comparison with C_1 group(Fig. 1and Table 2). In T_2 group with same concentration of monolaurin, the number of *B. cereus* was not significantly affected in comparison with C_2 group (Fig. 1 and Table 2). In C_2 group, starter culture decreased the count of *B. cereus* by 2.9 logs in comparison with C_1group (Table 2). In contrary, the combination of starter culture with monolaurin in T_2 group increased the number of *B. cereus* by 0.6 logs in comparison with C_2 group (Fig. 3). The effects of different storage times on *B.cereus* counts in the four groups of manufactured cheese are given in Table 3. Only in groups C_2 and T_2,as the storage times increased, the number of *B.cereus*was reduced significantly (Fig. 2).Comparison of *B.cereus* count affected by different storage days in the manufactured cheese samples showed a significant bacterial reduction in all days (1, 3, 5 and 7) except for day 0(p<0.05; Table 3).

Table 1. Changes in the mean numbers of *B. cereus* affected by various concentrations of monolaurin for a definite storage time and changes in the mean numbers of *B. cereus* affected by different storage times for a definite concentration of monolaurin in the four groups of cheese.

Cheese group	Monolaurin (ppm)	Number of *B.cereus* (log cfug^{-1}) ± SEM in different storage days					*p* value
		0	1	3	5	7	
C_1	0	$7.55^{c} \pm 0.95$	$7.82^{d} \pm 0.35$	$7.73^{c} \pm 0.30$	$7.38^{c} \pm 0.37$	$7.69^{c} \pm 0.71$	0.95
	100	$7.32^{c} \pm 0.13$	$7.81^{d} \pm 0.38$	$7.39^{bc} \pm 0.57$	$6.9^{c} \pm 0.7$	$7.11^{bc} \pm 0.56$	0.77
	200	$7.24^{c} \pm 0.16$	$7.51^{cd} \pm 0.19$	$7.54^{c} \pm 0.33$	$7.21^{c} \pm 0.35$	$7.01^{bc} \pm 0.44$	0.73
	400	$7.50^{c} \pm 0.1$	$7.37^{bcd} \pm 0.17$	$7.07^{bc} \pm 0.42$	$7.29^{c} \pm 0.36$	$6.77^{bc} \pm 0.47$	0.6
T_1	800	$6.51^{bc} \pm 0.04$	$7.05^{bcd} \pm 0.23$	$6.6^{bc} \pm 0.17$	$5.95^{ab} \pm 0.56$	$5.85^{bc} \pm 0.33$	0.12
	1200	$5.86^{b} \pm 0.46$	$5.71^{abc} \pm 0.41$	$5.57^{ab} \pm 0.24$	$5.54^{ab} \pm 0.25$	$5.06^{ab} \pm 0.23$	0.55
	1600	$4.68^{a} \pm 0.36$	$5.62^{ab} \pm 0.77$	$4.51^{a} \pm 0.72$	$4.62^{a} \pm 0.14$	$3.53^{a} \pm 0.69$	0.25
	2000	$4.56^{a} \pm 0.17$	$4.9^{a} \pm 0.39$	$3.83^{a} \pm 0.15$	$4.2^{a} \pm 0.34$	$3.31^{a} \pm 0.12$	0.01^{*}
C_2	0	$5.3^{ab} \pm 0.28$	$5.31^{a} \pm 0.15$	$5.09^{a} \pm 0.41$	$4.48^{a} \pm 0.16$	$3.53^{a} \pm 0.76$	0.06
	100	$6.45^{ab} \pm 0.33$	$6.21^{a} \pm 0.26$	$5.81^{a} \pm 0.46$	$5.05^{a} \pm 0.6$	$4.7^{a} \pm 0.41$	0.07
	200	$6.5^{ab} \pm 0.37$	$5.67^{a} \pm 0.18$	$5.31^{a} \pm 0.08$	$5.56^{a} \pm 0.42$	$5.22^{a} \pm 0.34$	0.09
	400	$6.7^{b} \pm 0.15$	$5.8^{a} \pm 0.44$	$5.32^{a} \pm 0.45$	$5.13^{a} \pm 0.29$	$5.31^{a} \pm 0.26$	0.05
T_2	800	$5.79^{ab} \pm 0.82$	$5.77^{a} \pm 0.6$	$6.15^{a} \pm 0.74$	$5.4^{a} \pm 0.27$	$4.96^{a} \pm 0.12$	0.67
	1200	$6.17^{ab} \pm 0.81$	$5.8^{a} \pm 1.04$	$5.73^{a} \pm 1.05$	$4.54^{a} \pm 1.17$	$4.78^{a} \pm 0.13$	0.68
	1600	$4.57^{ab} \pm 0.15$	$4.62^{a} \pm 0.2$	$3.96^{a} \pm 0.34$	$4.38^{a} \pm 0.26$	$4.2^{a} \pm 0.21$	0.35
	2000	$4.31^{a} \pm 0.15$	$4.52^{a} \pm 0.41$	$4.77^{a} \pm 0.25$	$4.52^{a} \pm 0.17$	$4.92^{a} \pm 0.51$	0.73

Means within the same column with different superscript differ significantly (p<0.05). Asterisk indicates significant differences in each row (p<0.5). C_1: without starter culture and monolaurin, T_1: without starter culture; with monolaurin, C_2: with starter culture; without monolaurin, T_2: with starter culture and monolaurin

Table 2. Changes in the mean numbers of *B. cereus* affected by various concentrations of monolaurinin the manufactured cheese samples during 7 days of storage at +7°C.

Monolaurin (ppm)	Number of *B. cereus*(log cfug^{-1}) ± SEM	
	Cheese with starter culture	Cheese without starter culture
0	4.74^{abc} ± 0.24	7.63^{d} ± 0.16
100	5.64^{c} ± 0.24	7.30^{d} ± 0.20
200	5.65^{c} ± 0.16	7.30^{d} ± 0.13
400	5.65^{c} ± 0.19	7.20^{d} ± 0.14
800	5.61^{bc} ± 0.24	6.39^{c} ± 0.16
1200	5.40^{bc} ± 0.38	5.55^{b} ± 0.14
1600	4.34^{a} ± 0.11	4.59^{a} ± 0.28
2000	4.60^{ab} ± 0.13	4.18^{a} ± 0.17

Means within the same column with different superscript differ significantly (p<0.05).

Table 3. Changes in the mean numbers of *B. cereus* affected by monolaurin in the manufactured cheese samples during various storage times at +7°C.

Day	Number of *B. cereus* (log cfug^{-1}) ± SEM	
	Cheese with starter culture	Cheese without starter culture
0	5.72^{b} ± 0.22	6.40^{a} ± 0.25
1	5.46^{ab} ± 0.18	6.72^{a} ± 0.25
3	5.27^{ab} ± 0.21	6.28^{a} ± 0.31
5	4.88^{a} ± 0.17	6.15^{a} ± 0.27
7	4.70^{a} ± 0.16	5.79^{a} ± 0.35

Means within the same column with different superscript differ significantly (p<0.05).

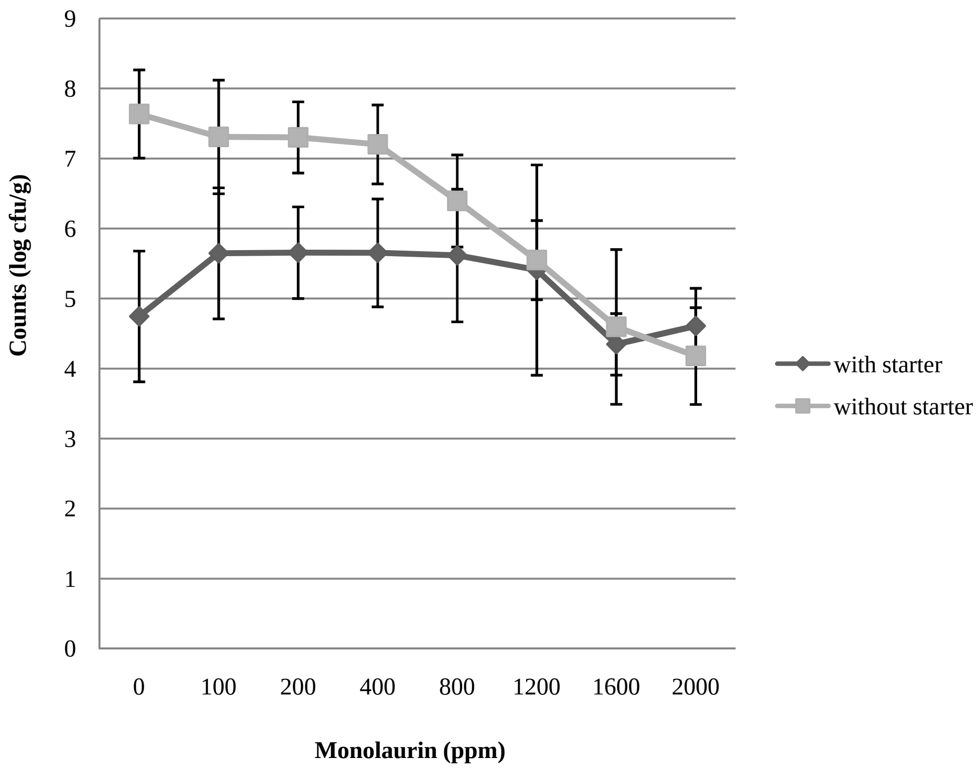


Figure 1. Comparing changes in the number of *B. cereus* affected by various concentrations of monolaurin in the manufactured cheese samples during 7 days of storage at +7°C.

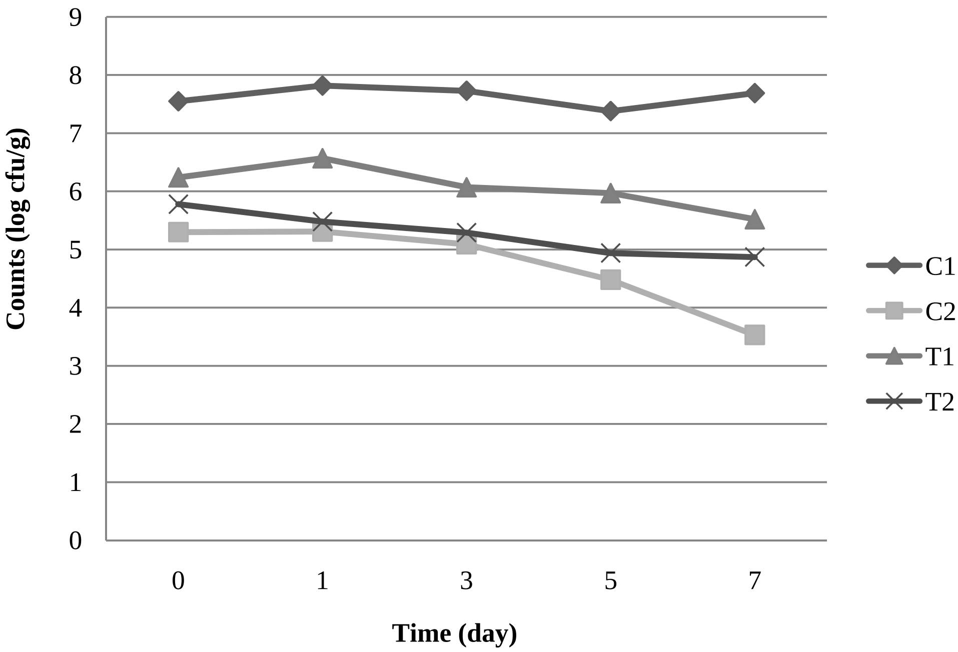


Figure 2. Comparing changes in the number of *B. cereus* affected by monolaurin in the manufactured cheese samples during various times of storage at +7°C. C_1**: without starter culture and monolaurin,** T_1**: without starter culture; with monolaurin,** C_2**: with starter culture; without monolaurin,** T_2**: with starter**

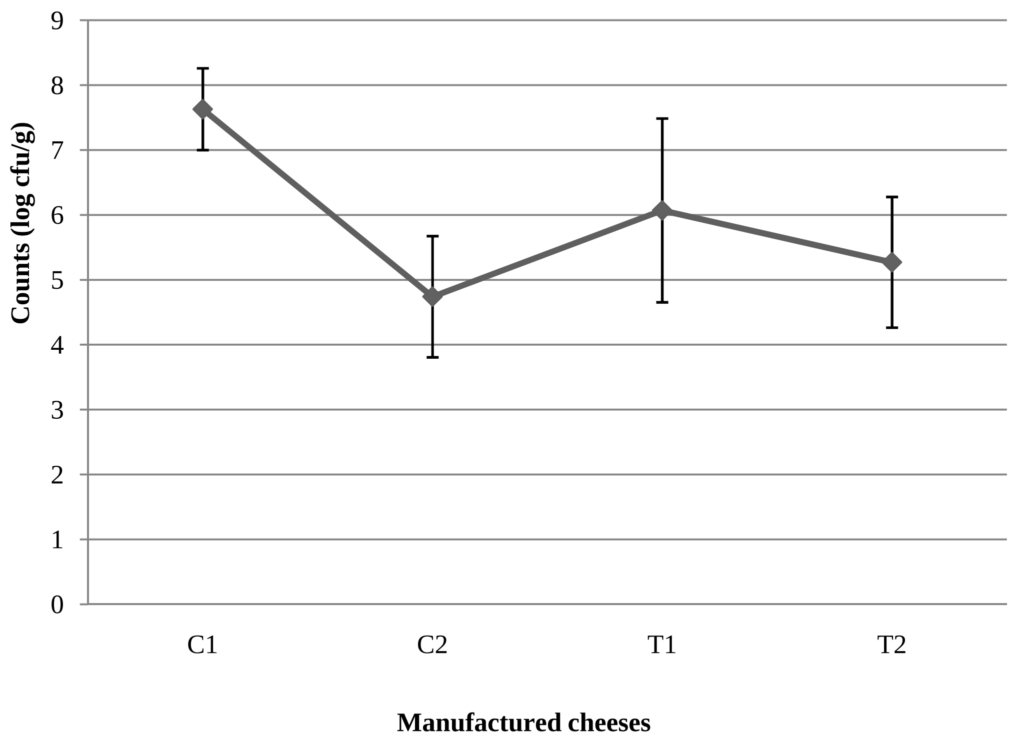


Figure 3. Changes in the total number of *B. cereus* in the four groups of manufactured cheese affected by different concentration of monolaurin during 7 days storage at +7°C.

Discussion

Antimicrobial activity of monolaurin against gram- positive bacteria has been well approved in vitro conditions. As well, it has been shown that monolaurin is effective on gram- negative bacteria only in presence of chelator agents such as EDTA and sodium citrate (Kabara, 1993, Razavi-Rohani and Griffiths, 1994 and 1996, Blaszyk *et al*., 1998, Branen and Davidson, 2004 and Preuss *et al*, 2005). There is a little published data about antibacterial effects of monolaurin separately and in combination with lactic acid bacteria (LAB) starter culture in food systems, especially dairy products.

Ababouch *et al* (1994) reported that the growth of spores and vegetative cells of *B. cereus* is inhibited by fatty acids and monolaurin. Also, Cotton and Marshall (1997) showed that monolaurin is more effective on vegetative cells of *B. cereus* when dissolved in ethanol than when heat-dispersed in an aqueous system. In a study, microbial shelf-life of cottage cheese in presence of monolaurin increased approximately 5-10 days during storage at +6°C (Bautista *et al*., 1993). In another study, the synergistic inhibitory effects of nisin and momolaurin on vegetative cells of *B. cereus* in milk during 5 days storage at 37°C were reported (Mansour and Milliere, 2001).

According to Wong and Chen (1988) growth of vegetative cells of *B. cereus* were not affected by lactic acid bacteria at the beginning of the fermentation in non-fatty milk, but were affected strongly with continued fermentation. Work by Byaruhanga *et al* (1999) on the growth and survival of *B. cereus* in mageu, a sour maize beverage, showed that starter culture fermentation and development of acidity are major factors in inhibiting the growth of this bacterium. Rukure and Bester (2001) concluded that *B. cereus* spores could germinate and grow into vegetative cells during the early stage of Gouda cheese manufacturing, but the vegetative cells were not able to survive during the final stages of the manufacturing process and ripening of the cheese. Rossland *et al* (2003) reported that lactococcus and lactobacillus starter cultures had the inhibitory effects on the growth of *B. cereus* in milk. Furthermore, Yang *et al* (2007) showed that lactic acid bacteria (LAB) starter cultures used in rice fermentation inhibited spores and vegetative cells of *B. cereus*.

In the present study, monolaurin showed the inhibitory effects on the growth of *B. cereus*cells in cheese samples of the group T_1 which is similar to the results obtained by Ababouch *et al* (1994), Cotton and Marshall (1997) and Mansour and milliere (2001). In addition, the inhibition of *B. cereus* cells by lactic acid bacteria starter culture in cheese samples of the group C_2 is in accordance with the results found by Wong and Chen (1988a), Byaruhanga *et al* (1999), Rukure and Bester (2001), Rossland *et al* (2003) and Yang *et al* (2007). However, the result demonstrated that in cheese samples of the group T_2, the combination of monolaurin with starter culture did not show the inhibitory synergistic effects on the growth of *B. cereus* cells in comparison with cheese sample of the group C_2. It can be concluded that the combination of monolaurin with starter culture resulted in inhibition of starter culture and prevented its effects on lactic acid production and inhibition of *B. cereus* cells. Therefore, simultaneous use of monolaurin with starter culture is not recommended for improving the microbial shelf-life of Iranian white fresh cheese.

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مطالعه اثرات مونولورین و کشت آغازگر لاکتیکی روی رشد سلول های رویان *باسیلوس سرئوس* درپنیرسفید تازه ایرانی

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چکیده

با توجه به اثرات مضر نگه دارنده‌های شیمیایی غذایی، استفاده از نگه دارنده‌های طبیعی در حال گسترش می‌باشد. مونولورین یک مونواستر اسید لوریک بوده و به طور طبیعی در بعضی از غذاها یافت شده و دارای اثرات ضد ویروسی و ضد باکتریایی مختلفی می‌باشد. ارزیابی اثرات مونولورین به تنهایی و در ترکیب با کشت آغازگر لاکتیکی روی رشد سلول‌های رویای *باسیلوس سرئوس* ATCC۱۱۷۷۸ در پنیرهای تولیدی از اهداف این تحقیق بودند. در این مطالعه، تعداد *باسیلوس سرئوس* در چهار گروه پنیر (C_1: بدون کشت آغازگر و بدون مونولورین،C_2: با کشت آغازگر و بدون مونولورین، T_1: بدون کشت آغازگر و با مونولورین، T_2: با کشت آغازگر و با مونولورین) در روزهای صفر، یک، سه، پنج و هفت تولید شمارش شدند. در گروه T_1، مونولورین در غلظت های ۸۰۰، ۱۲۰۰، ۱۶۰۰ و ppm۲۰۰۰ تعداد *باسیلوس سرئوس* را در مقایسه با گروه C_1، به ترتیب ۱/۲، ۲/۱، ۳ و ۳/۴ لگاریتم کاهش داد. در گروه T_2، تعداد *باسیلوس سرئوس* در مقایسه با گروه C_2، به صورت معنی داری تحت تاثیر قرار نگرفت ($p>0.05$). در گروه C_2، کشت آغازگر تعداد *باسیلوس سرئوس* را در مقایسه با گروه C_1، ۲/۹ لگاریتم کاهش داد. در مقابل ترکیب کشت آغازگر با مونولورین در گروه T_2، تعداد *باسیلوس سرئوس* را در مقایسه با گروه C_2، ۰/۶ لگاریتم افزایش داد. همچنین در گروه های C_2 و T_2، تعداد *باسیلوس سرئوس* با افزایش مدت نگهداری کاهش یافت. از نتایج می‌توان استنباط نمود که در نمونه پنیرهای گروه T_1، مونولورین به تنهایی اثرات مهاری روی رشد سلول‌های *باسیلوس سرئوس* نشان داد در حالی‌که در نمونه پنیرهای گروه T_2، ترکیب مونولورین با کشت آغازگر اثرات مهاری سینرژیستی روی سلول‌های این باکتری نشان نداد. بنابراین برای بالا بردن ماندگاری میکروبی پنیر سفید تازه ایرانی استفاده همزمان مونولورین با کشت آغازگر پیشنهاد نمی‌گردد.

واژگان کلیدی: مونولورین، کشت آغازگر، *باسیلوس سرئوس*، پنیرسفید تازه ایرانی